High-Performance Liquid Chromatography of Nucleobases, Nucleosides and Nucleotides

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOBASES, NUCLEOSIDES AND NUCLEOTIDES

I. MOBILE PHASE COMPOSITION FOR THE SEPARATION OF CHARGED SOLUTES BY REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

To find optimal conditions for the separation of nucleobases, nucleosides and nucleotides by reversed-phase chromatography, the polarity, pH, ion concentration and polarity of the buffer ions of the mobile phase were varied. A systematic study of the effects of these parameters on retardation led to the formulation of the following simple rules: (1) methanol changes only column capacity ratios, not selectivity; (2) pH influences both column capacity ratios and selectivity; (3) the concentration of buffer ions had hardly any effect on retardation; (4) the hydrophobicity of the buffer ions has strong effects only on column capacity ratios and selectivity of solutes with opposite charge; and (5) a mixture of buffer ions with different hydrophobicities has the chromatographic properties of a buffer ion with intermediate hydrophobicity.

INTRODUCTION

Nucleobases and nucleosides have been separated by standard methods on cation exchangers at acidic pH[1] and nucleotides on anion exchangers at neutral or basic pH[2]. Nucleobases and nucleosides have also been separated on anion exchangers at basic pH[3,4], and under these conditions the simultaneous separation of all compounds on one column was possible[5,6]. The introduction of reversed phases facilitated this simultaneous separation[7,8], especially when hydrophobic buffer ions were used[9]. More recently, affinity chromatography of nucleobases has been reported, using immobilized thymidine as the stationary phase[10]. This class of substances can be separated successfully under many different conditions[1–30] owing to their intrinsic physical properties, combining acidic, basic, polar and lipophilic moieties within one chemical structure.

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To optimize chromatographic conditions, one should use all of these physical properties. It is important to know the effects of different mobile phase parameters such as polarity, pH, ion concentration and polarity of the buffer ions on the retardation of solutes with different physical properties.

The relationship between the resolution of two solutes and selectivity and column capacity ratios can be formulated as[31]

$$R_s = \frac{1}{2} \cdot \frac{\overline{k'}}{\overline{k'} + 1} \cdot \frac{1 - \alpha}{1 + \alpha} \left(\frac{L}{H}\right)^{\frac{1}{2}}$$

where R_s = resolution, $\overline{k'}$ = mean column capacity ratio, α = selectivity, L = length of the column and H = height equivalent to a theoretical plate. Although H is not independent of k'[32], this equation provides a theoretical guide to the optimal composition of mobile phases in chromatography. In general, it is easier to increase k' values than α values. k' values above about 4 are used only if many compounds have to be separated, because the resolution is already at 80% of its maximal value. If the resolution is insufficient, the mobile phase should be changed in such a way that the selectivity (α) is more affected than column capacity ratios (k'). In practice, the optimal separation conditions should combine small column capacity ratios with high selectivity. Therefore, the effects of the polarity of the mobile phase, its pH, ion concentration and the polarity of the cations on column capacity ratio and selectivity were studied systematically on a reversed-phase column.

EXPERIMENTAL

The high-performance liquid chromatographic (HPLC) equipment consisted of an Altex Model 100A pump, a Rheodyne 7105 injector, a reversed-phase column (5 μm, RP-8, Riedel-de Haën, Hannover, G.F.R.; self-packed, 300 × 3 mm I.D.) and a Perkin-Elmer LC-55 variable wavelength detector set at 260 nm. Retention times were recorded with a stopwatch and were reproducible with a standard deviation of 0.5%. The ion concentrations of the mobile phase are always given for the cations and the total volume. The pH was measured with an E-516 Titriskop Metrohm Herisau pH meter, and is given for the final mobile phase composition. The flow-rate was 1.5 ml/min. The pressure varied with the composition of the mobile phase between 110 and 150 bar. All experiments were carried out at room temperature.

RESULTS AND DISCUSSION

In addition to the solutes listed in Table I*, the following compounds were investigated the same way (data not shown): 8-methoxy-cAMP (B), 8-hydroxy-cAMP (B), 8-bromo-cAMP (B), 5-aminoimidazole-4-carboxamide-1-ribose-3′,5′-monophosphate (AICAR) (B), xanthosine-3′,5′-monophosphate (B), adenosine-N^1-

* Abbreviations used: cAMP = adenosine-3′,5′-monophosphate; 5′-AMP = adenosine-5′-monophosphate; cPMP = purine-riboside-3′,5′-monophosphate; B = Boehringer (Mannheim, G.F.R.); S = Sigma (St. Louis, MO, U.S.A.); P = Pharma-Waldhof (Mannheim, G.F.R.); M = Merck (Darmstadt, G.F.R.).

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF SOLUTES

No.	*Compound*	*Source*	*Hydrophobicity* at pH 3.0*	*Electrophoretic mobility** at pH 3.0*
1	cAMP	P	0.8	−3.1
2	N^6-Monobutyryl-cAMP	B	3.9	−8.4
3	6-Chloro-cPMP	B	2.0	−10.8
4	8-Amino-cAMP	B	0.6	+0.5
5	8-Dimethylamino-cAMP	B	3.5	−0.1
6	8-Benzylamino-cAMP	B	11.2	+0.7
7	5′-AMP	B	0.3	−2.9
8	Adenosine	M	1.1	+11.1
9	Adenine	B	0.8	+19.5

* Hydrophobicity is expressed as the k' value on the reversed-phase column in 10% methanol–10 m*M* ammonium formate (pH 3.0).

** The electrophoretic mobility is expressed as the rate of movement in cm/h of the solutes to the negative pole (positive sign) or positive pole (negative sign) of a Pherograph high-voltage electrophoretic apparatus. The electrophoretic movement is divided by the R_F value of the solute in ascending paper chromatography, to correct for adsorption of the solutes to the paper.

oxide (S), 2′-deoxyadenosine (M), 5′-tosyladenosine (P), 2′,3′-isopropylideneadenosine (S), 2′,3′-O-*p*-methoxybenzylideneadenosine (S), guanosine (P), 2′-deoxyguanosine (M), cytidine (P), 2′-deoxycytidine (P), uridine (P), thymidine (B) and purine riboside (S). The compounds in Table I were chosen for the following reasons:

(1) cAMP, adenosine and adenine have similar polarities but different charges;

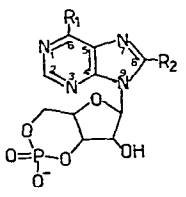
(2) cAMP and 5′-AMP have identical charges at pH 3.0 but differ in polarity;

(3) 8-amino-cAMP, 8-dimethylamino-cAMP and 8-benzylamino-cAMP have similar charges but their polarities differ widely;

(4) N^6-monobutyryl-cAMP and 6-chloro-cPMP were chosen as the least protonated solutes at pH 3.0.

The structures of these cyclic nucleotide derivatives are shown in Fig. 1.

The electrophoretic mobilities of the solutes were determined in 0.04 *M* citrate–hydrochloric acid (pH 3.0) on Whatman 3MM paper using Pherograph high-voltage electrophoresis at 2000 V. Adsorption of the solutes to this paper was determined in this buffer using ascending paper chromatography. As an indication of the charge of the solutes at pH 3.0, the electrophoretic mobility was divided by the R_F value for paper chromatography.



No	R_1	R_2
1	$-NH_2$	$-H$
2	$-NH-CO-CH_2-CH_2-CH_3$	$-H$
3	$-Cl$	$-H$
4	$-NH_2$	$-NH_2$
5	$-NH_2$	$-N(CH_3)_2$
6	$-NH_2$	$-NH-CH_2-C_6H_5$

Fig. 1. Structures of the cyclic nucleotide derivatives.

The silanol groups that are still present after chemical modification of the silica gel matrix often have no influence on retardation[33], and this occurred with this column; retention times of polar compounds did not increase at higher methanol concentrations (data not shown). Therefore, it is assumed that all chromatographic effects are due to interactions between components of the mobile phase, the solutes and the octyl moieties of the stationary phase. In most experiments the pH was kept at 3.0, as at this pH most compounds are partially protonated (Table I). Different substituted ammonium ions were used to give a specific hydrophobicity[34].

Polarity of the mobile phase

An increase in methanol concentration decreases the retention times in reversed-phase chromatography (Fig. 2). The effect of the hydrophobic constituents at the adenine moiety on column capacity ratios is shown in the order of elution of 8-amino-cAMP (4), 8-dimethylamino-cAMP (5) and 8-benzylamino-cAMP (6). The methanol concentration has virtually no effect on selectivity, probably indicating that

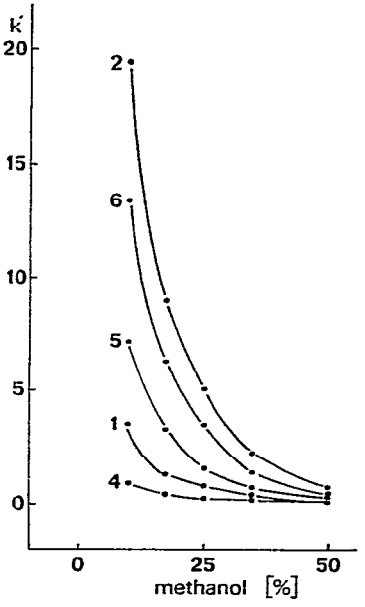


Fig. 2. Influence of methanol concentration on column capacity ratios. Mobile phase: 10 m*M* tributylammonium formate (pH 3.0). Solutes (see Table I): 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 4 = 8-amino-cAMP; 5 = 8-dimethylamino-cAMP; 6 = 8-benzylamino-cAMP.

methanol does not modify the type but rather the intensity of interaction between solutes and the stationary phase; it competes with the solute for occupation of the octyl groups.

As methanol changes only k' values, a decrease in methanol concentration does not result in a much improved resolution of two solutes with small selectivity, but in a longer analysis time.

pH of the mobile phase

The pH of the mobile phase may affect the polarity of the solutes more strongly than the polarity of the mobile and stationary phases. Protonation of basic groups of the solutes results in an increase in polarity, and therefore in a decrease in retention time. The pK value of cyclic AMP (1) is approximately 3.4 (Table I), which agrees well with the significant change in k' values between pH 3 and 4 (Fig. 3). Substitution of the N^6-amino group in N^6-monobutyryl-cAMP (2) decreases the pK value to approximately 2.5 (Table I), and shifts the pH-sensitive part of the curve to values below pH 3.0. By removal of the amino group as in 6-chloro-cPMP (3), no protonation occurs (Table I) and the pH has little effect on retardation. Hence the pH of the mobile phase only influences the k' values of those solutes which have a pK value in the region where the pH was changed. Because most compounds have different pK values, this parameter is particularly useful in modifying selectivity.

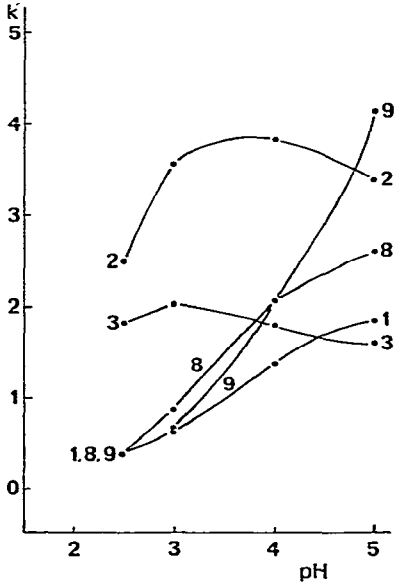


Fig. 3. Influence of pH on column capacity ratios. Mobile phase: 10% methanol–10 mM ammonium formate. Solutes (see Table I): 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 3 = 6-chloro-cPMP; 8 = adenosine; 9 = adenine.

Ion concentration of the mobile phase

The concentration of the buffer ions was varied between 0.001 and 0.1 M. Solutes were injected at a relatively low concentration of 1 μM. Changes in ion concentration may change the equilibrium of the solutes in ion-paired and non-paired forms. The concentration of buffer ions also may change the polarity of the mobile phase[35,36]. Fig. 4 shows that the ion concentration has noticable but small effects on retardation and selectivity on a reversed-phase column. This parameter is therefore of minor importance for optimizing mobile phase compositions for a reversed-phase column.

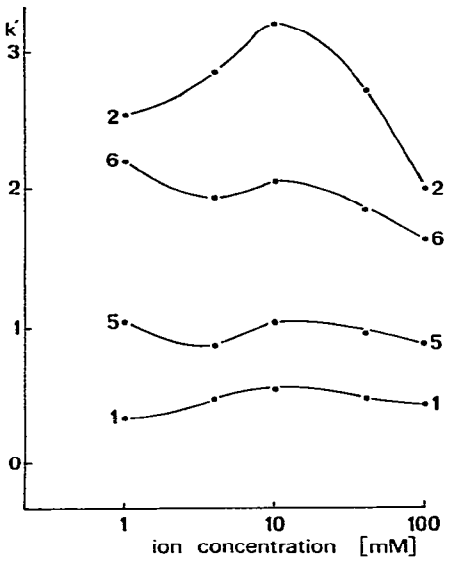


Fig. 4. Influence of concentration of buffer ions on column capacity ratios. Mobile phase: 30% methanol–tributylammonium formate (pH 3.0). Solutes: (see Table I): 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 5 = 8-dimethylamino-cAMP; 6 = 8-benzylamino-cAMP.

Hydrophobicity of the buffer ions

Increasing hydrophobicity of the cations results in an increase in the k' values of negatively charged solutes [Fig. 5A, cyclic AMP (1) and 5'-AMP (7)] and a slight decrease in the k' values of positively charged solutes [adenosine (8) and adenine (9) in Fig. 5A].

Retardation of the cyclic AMP derivatives with a distinct net negative charge at pH 3.0 is strongly influenced by increasing hydrophobicity of the cations [N^6-monobutyryl-cAMP (2) and 6-chloro-cPMP (3) in Fig. 5B]. The retention times of the cyclic AMP derivatives with a close to zero net charge, such as 8-amino-cAMP (4), 8-dimethylamino-cAMP (5) and 8-benzylamino-cAMP (6), are only slightly influenced by the polarity of the buffer ions (Fig. 5B). These results demonstrate the anion-exchange properties of a reversed-phase column in the presence of hydrophobic buffer cations.

Reversed-phase interactions are involved in the retardation mechanism, as is shown by the constant differences in the k' values of 8-amino-cAMP (4), 8-dimethylamino-cAMP (5) and 8-benzylamino-cAMP (6). A lively discussion is going on in the literature on the mechanism by which charged solutes are retarded on reversed-phase matrices in the presence of hydrophobic ions. The result is a variety of terms for this type of chromatography, such as "soap chromatography"[37,38], "ion-pair chromatography"[39], "solvent-generated dynamic ion-exchange chromatography"[40,41], "hetaeric chromatography"[42], "paired-ion chromatography"[43], "detergent-based cation exchange"[40], "solvophobic-ion chromatography"[9], "surfactant chromatography "[44] and "ion interaction"[45]. This variety indicates the uncertainty that exists concerning the retention mechanism of this type of chromatography. For the practice of chromatography it may be sufficient to state that increasing hydrophobicity of

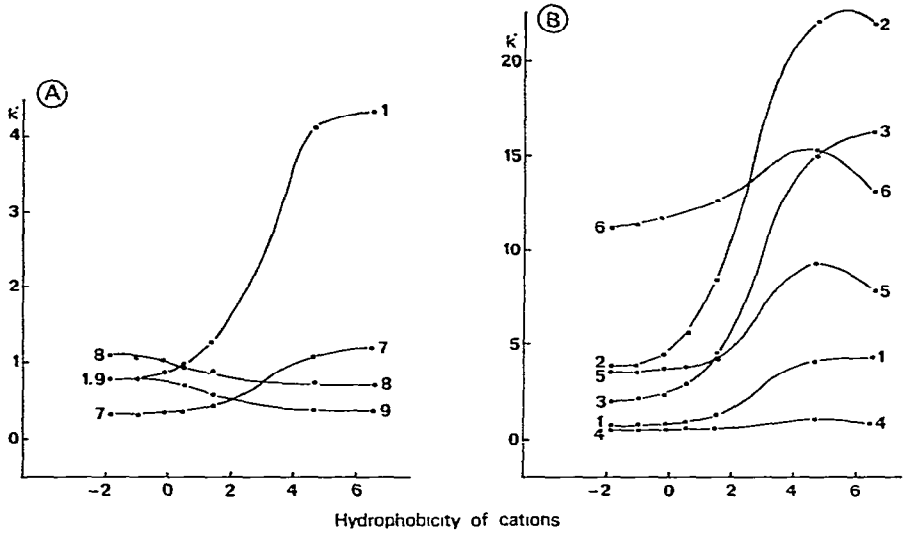


Fig. 5. Influence of the hydrophobicity of the cations on column capacity ratios. Abscissa: hydrophobicity of the cations, calculated with hydrophobic fragmental constants[34]. Cations from left to right on each curve: ethanolammonium, ammonium, monoethylammonium, diethylammonium, triethylammonium, tributylammonium and tetrabutylammonium. Mobile phase: 10% methanol–10 m*M* cations (pH 3.0), adjusted with formic acid. Solutes: see Table I and Fig. 1.

buffer ions results in unchanged retardation of solutes without charge, in unchanged or diminished retardation of solutes with the same charge as the modifying buffer ion, and in stronger retardation of solutes with an opposite charge of the modifying buffer ion.

Mixture of buffer ions with different hydrophobicities

The change of a buffer ion by subsequent substitutions through hydrophobic groups can only result in discrete values of the hydrophobicity of the buffer ions. It will be time consuming to find the specific buffer ion which has the desired hydrophobicity. A mixture of two buffer ions with different hydrophobicities may act as a buffer ion with intermediate hydrophobicity. In Fig. 6 the ratio of concentrations of two cations with different hydrophobicities is varied; the change in selectivity is comparable to the change in Fig. 5, where the hydrophobicity of only one cation was varied. A comparison of Figs. 5 and 6 shows that a mixture such as 10 m*M* tributylammonium formate plus 100 m*M* ammonium formate results in a similar slectivity to 10 m*M* triethylammonium formate.

Mixtures of buffer ions with different hydrophobicities can provide a continuous range of hydrophobicities, and it is easier to find an optimal mixture than a cation with optimal hydrophobicity.

Practical aspects of reversed-phase chromatography

The action of mobile phase components on the retardation of charged solutes by reversed-phase chromatography can be summarized by the following simple rules:

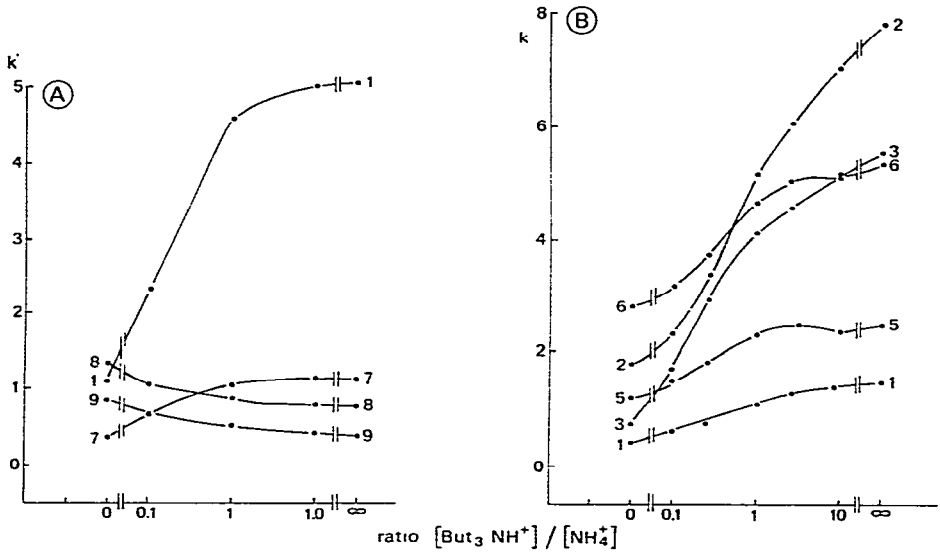


Fig. 6. Influence of a mixture of cations with different hydrophobicities on column capacity ratios. Abscissa: ratio of tributylammonium (But_3NH^+) and ammonium (NH_4^+) concentrations; this ratio was produced by mixing 10 m*M* tributylammonium formate with different concentrations ammonium formate, except where the ratio is 0 or ∞ which were obtained by applying 10 m*M* ammonium formate or 10 m*M* tributylammonium formate, respectively. A, 5% methanol (pH 3.0); B, 25% methanol (pH 3.0). Solutes: see Table I and Fig. 1.

(1) Methanol reduces only the column capacity ratios, without having a strong effect on selectivity.

(2) The pH of the mobile phase influences both column capacity ratios and selectivity. Its action depends mainly on the p*K* values of the solutes.

(3) The concentration of buffer ions has only minor effects on column capacity ratios and selectivity.

(4) The hydrophobicity of a buffer ion has pronounced effects on column capacity ratios and the selectivity of solutes with opposite charge, and small effects on the retardation of solutes without charge or the same charge as the buffer ions.

(5) A mixture of buffer ions with different hydrophobicities acts as a buffer ion with intermediate hydrophobicity. The hydrophobicity of a mixture is determined by the ratio of the concentrations of the components rather than by their absolute concentrations.

During the last 2 years we have applied these rules to several separation problems using different columns. For our studies on the degradation of cyclic nucleotide derivatives by a liver homogenate[46], simultaneous separation of nucleotides, nucleosides and nucleobases was desirable. For the separation of degradation products of 2′-(2,4-dinitrophenoxy)-cAMP, a reversed-phase column was chosen because of the high hydrophobicity of the dinitrophenoxy moiety. A relatively high pH was used in order to discriminate between the charge of phosphate diesters (cAMP) and phosphate monoesters (5′-AMP). Phosphate buffer has a good buffering capacity at pH 6, whereas the ion concentration is not important. First, the methanol concentration was varied to obtain a preliminary separation, then the selectivity between the group

of nucleosides and the group of nucleotides was improved by adding a small amount of tributylammonium formate. Finally, the optimum compromise between resolution and analysis time was found by variation of the methanol concentration (Fig. 7A).

The appearance of 2′-(2,4-dinitrophenoxy)-5′-AMP among the degradation products of this cyclic nucleotide derivative was surprising, as 5′-AMP did not appear as one of the degradation products of cyclic AMP. To prove that the compound which forms peak 3 has a negative charge, more tributylammonium formate was added to the mobile phase. The nucleotides disappeared from the chromatogram, whereas the retention time of the nucleoside remained unchanged (Fig. 7B).

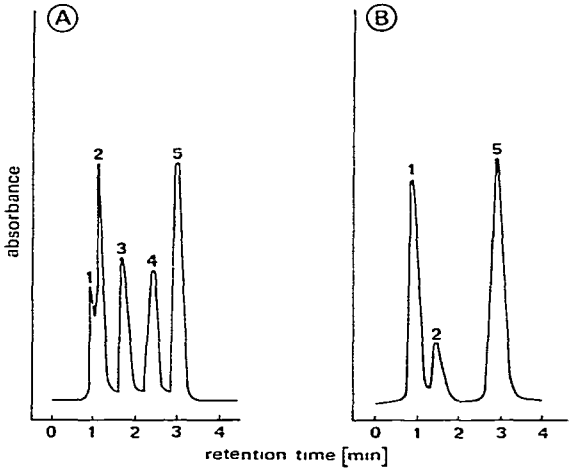


Fig. 7. Separation of 2′-(2,4-dinitrophenoxy)-cAMP incubated with a liver homogenate for 8 h[46]. Stationary phase: RP-18 (Machery, Nagel & Co., Düren, G.F.R.), 300 × 3 mm. Mobile phase: A, 8 m*M* Na_2HPO_4/H_3PO_4–1 m*M* tributylammonium formate–48% methanol (pH 6.6); B, 8 m*M* Na_2HPO_4/H_3PO_4–10 m*M* tributylammonium formate–48% methanol (pH 6.6). Peaks: 1 and 2 = UV-absorbing compound from the liver homogenate; 3 = 2′-(2,4-dinitrophenoxy)-5′-AMP; 4 = 2′-(2,4-dinitrophenoxy)-cAMP; 5 = 2′-(2,4-dinitrophenoxy)inosine.

Another example is kinetic studies of cyclic nucleotide phosphodiesterase under non-equilibrium conditions which require the separation of minute amounts of product from large amounts of substrate. Because peak tailing occurs more often than an extended front of the peak, the high substrate peak should be the last one in the chromatogram. As enzyme preparations are rarely pure, the further degradation products should also be analysed. For cyclic nucleotide phosphodiesterase the optimal separation would take place when all nucleosides and 5′-AMP coincide in one peak just after the injection peak and cyclic AMP appears later in the chromatogram. A reversed phase gives the correct order of elution of 5′-AMP and cyclic AMP. Cyclic AMP becomes the most lipophilic compound on using triethylammonium or tributylammonium formate as the mobile phase buffer (*cf.*, Fig. 5A).

The pH should be below 5, otherwise 5′-AMP acquires two negative charges and therefore two lipophilic counter ions. With triethylammonium formate as the buffer ion adenosine appeared after 5′-AMP, whereas with tributylammonium formate 5′-AMP appeared after adenosine. By mixing different concentrations of trieth-

ylammonium with tributylammonium ions a mixture was obtained that resulted in identical retardation times of adenosine and 5′-AMP. Separation between this peak and cyclic AMP was optimized by variation of the methanol concentration (Fig. 8A).

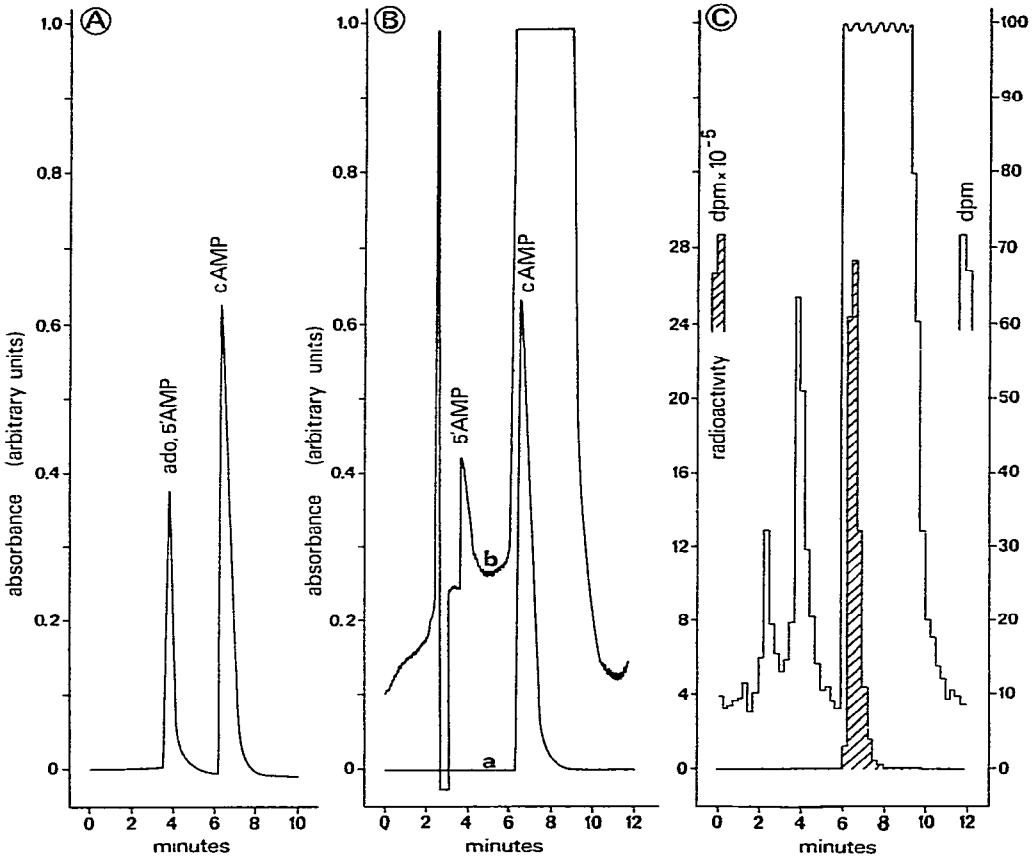


Fig. 8. Separation of large amounts of cyclic AMP from minute amounts of 5′-AMP, adenosine or further degradation products. Equipment: Altex Model 100A high-pressure pump, Valco 7000 p.s.i. injector, Laboratory Data Control UVIII (1203) detector (254 nm) and a Kipp BD 40 flat-bed recorder. Stationary phase: reversed-phase LiChrosorb 5RP-18 (250 cm × 4.6 mm) with Vydac 201 SC pre-column (100 cm × 2.1 mm). Mobile phase: 5 m*M* triethylammonium formate–0.5 m*M* tributylammonium formate–25% methanol (pH 4.5) (adjusted with formic acid); flow-rate, 1 ml/min; pressure, 306 bar. (A) Separation of $0.5 \cdot 10^{-9}$ moles of adenosine, $0.5 \cdot 10^{-9}$ moles of 5′-AMP and $5 \cdot 10^{-9}$ moles of cyclic AMP. Injection volume: 10 μl. Sensitivity: detector at 0.128, recorder at 10 mV; thus, one arbitrary unit ≡ 0.128 absorbance unit. (B) Separation of 10^{-7} moles of cyclic AMP from $2 \cdot 10^{-12}$ moles of 5′-AMP. Injection volume: 100 μl. Sensitivity: a = detector at 2.048, recorder at 10 mV (one arbitrary unit ≡ 2.048 absorbance unit); b = detector at 0.002, recorder at 2 mV (one arbitrary unit ≡ 0.0004 absorbance unit). Cyclic AMP was purified previously under the same chromatographic conditions. (C) Separation of 10^{-10} moles of [^{3}H]cAMP from 10^{-15} moles of [^{3}H]-5′-AMP and possibly further degradation products: 10^{-10} moles of [2,8-^{3}H]cAMP (52 Ci/mmol; Radiochemical Centre, Amersham, Great Britain) were incubated in 25 m*M* Tris–HCl–2 m*M* $MgCl_2$ (pH 7.5) with $2.5 \cdot 10^{-10}$ g of cyclic nucleotide phosphodiesterase (Boehringer) in a total volume of 10 μl. Ten seconds after the initiation the reaction was stopped by injection of the incubation mixture on to the column. The eluent was divided into 0.25-ml fractions, the radioactivity of which was determined. [2,8-^{3}H]cAMP was purified previously under the same chromatographic conditions.

The background noise of the UV detector is approximately 0.00001 absorbance unit. Fig. 8B shows the separation of 10^{-7} moles of cyclic AMP from $2 \cdot 10^{-12}$ moles of 5'-AMP. By making use of radioactive cyclic AMP the sensitivity is determined by the specific activity of the radioactive label. Fig. 8C shows the separation of 10^{-10} moles of [^{3}H]cAMP from 10^{-15} moles of degradation products (5'-AMP and/or nucleosides). The detection limit can be reduced still further if [^{32}P]cAMP (1000–3000 Ci/mmole, NEG-011; New England Nuclear, Boston, MA, U.S.A.) is used. The detection limit would be then 10^{-17} moles, which is only 6 million molecules.

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